Sussex Research Online

Metabolic and physiological adjustment of Suaeda maritima to combined salinity and hypoxia

Article (Accepted Version)

Behr, Jan H, Bouchereau, Alain, Berardocco, Solenne, Seal, Charlotte E, Flowers, Timothy and Zörb, Christian (2017) Metabolic and physiological adjustment of Suaeda maritima to combined salinity and hypoxia. Annals of Botany, 119 (6). pp. 965-976. ISSN 0305-7364

This version is available from Sussex Research Online: http://sro.sussex.ac.uk/id/eprint/73393/

This document is made available in accordance with publisher policies and may differ from the published version or from the version of record. If you wish to cite this item you are advised to consult the publisher's version. Please see the URL above for details on accessing the published version.

Copyright and reuse:
Sussex Research Online is a digital repository of the research output of the University.

Copyright and all moral rights to the version of the paper presented here belong to the individual author(s) and/or other copyright owners. To the extent reasonable and practicable, the material made available in SRO has been checked for eligibility before being made available.

Copies of full text items generally can be reproduced, displayed or performed and given to third parties in any format or medium for personal research or study, educational, or not-for-profit purposes without prior permission or charge, provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way.

http://sro.sussex.ac.uk

Metabolic and physiological adjustment of *Suaeda maritima* to combined salinity and hypoxia

Jan Behr[1*], Alain Bouchereau[2], Solenne Berardocco[2], Charlotte E. Seal[3], Timothy Flowers[4,5], Christian Zörb[1]

[1] University of Hohenheim, Institute of Crop Science, Quality of Plant Products; Emil-Wolff-Str. 25, 70599 Stuttgart

[2] UMR1349 INRA, Agrocampus Ouest, Université de Rennes 1, F-35653, Le Rheu, France

[3] Comparative Plant and Fungal Biology, Royal Botanic Gardens, Kew, Wakehurst Place, Ardingly, West Sussex, RH17 6TN, UK

[4] School of Life Sciences, University of Sussex, Falmer, Brighton BN7 1BD, UK

[5]School of Plant Biology (M084), The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

**For correspondance. E-mail jan.behr@uni-hohenheim.de*

• **Background and Aims** *Suaeda maritima* is a halophyte commonly found on coastal wet lands in the intertidal zone. Due to its habitat *S. maritima* has evolved tolerance to high salt concentrations and hypoxic conditions in the soil caused by periodic flooding. In the present work, the adaptive mechanisms of *S. maritima* to salinity combined with hypoxia were investigated on a physiological and metabolic level.

• **Methods** To compare the adaptive mechanisms to deficient, optimal and stressful salt concentrations, *S. maritima* plants were grown in a hydroponic culture under low-, medium- and high-salt concentrations. Additionally, hypoxic conditions were applied to investigate the impact of hypoxia combined with different salt concentrations. A non-targeted metabolic approach was used to clarify the biochemical pathways underlying the metabolic and physiological adaptation mechanisms of *S. maritima*.

• **Key Results** Roots exposed to hypoxic conditions showed an increased level of tricarboxylic acid (TCA) -cycle intermediates such as succinate, malate and citrate. During hypoxia, the concentration of free amino acids increased in shoots and roots. Osmoprotectants such as proline and glycine betaine increased in concentrations as the external salinity was increased under hypoxic conditions.

• **Conclusions** The combination of high salinity and hypoxia caused an ionic imbalance and an increase of metabolites associated with osmotic stress and photorespiration, indicating a severe physiological and metabolic response under these conditions. A disturbed proline degradation in the roots induced an enhanced proline accumulation under hypoxia. The enhanced alanine fermentation combined with a partial flux of the TCA-cycle might contribute to the tolerance of *S. maritima* to hypoxic conditions.

Key words: *Suaeda maritima*, halophyte, metabolomics, salinity, hypoxia, osmotic adjustment,

INTRODUCTION

Salinity is an important limiting factor in agriculture, especially in areas that are extensively irrigated (Smedema and Shiati, 2002). High ion concentrations decrease the osmotic potential of the soil and reduce the availability of water for plant roots and nutrient use efficiency (Baligar *et al.*, 2007; Munns and Tester, 2008). The accumulation of sodium and chloride in the roots and shoots of salt-sensitive plants induces toxic effects by disturbing their essential cellular metabolism such as protein synthesis, enzyme activity and photosynthesis (Horie *et al.*, 2012). Moreover, salinity also disturbs K^+/Na^+ homeostasis in plants (Maathuis, 1999; Zörb *et al.*, 2014). The natural habitat of halophytes are saline soils, with euhalophytes characterized as plants completing their life cycle in high salt concentrations of $\geq$ 200 mM NaCl (Flowers *et al.*, 2015). A wide range of adaptive mechanisms allow halophytes to tolerate salt concentrations in soils that are damaging for salt-sensitive plants. These adaptive mechanisms include osmotic adjustment by the production of compatible solutes and the controlled uptake of salt, its transport from the root system through the xylem to the shoot, and the accumulation of salt into plant organs and organelles. Some species also use the secretion of salt and/or the succulence of plant organs as a tolerance mechanism to high salt concentrations. High salinity induces the accumulation of reactive oxygen species (ROS), which are scavenged by detoxifying compounds to prevent cellular damages (de Boer and Volkov, 2003; Ashraf and Harris, 2004; Flowers and Colmer, 2008; Alhdad *et al.*, 2013).

Saline soil can, additionally, be exposed to waterlogging because of shallow water-tables or decreased infiltration of surface water due to sodicity (Ghassemi *et al.*, 1995; Barrett-Lennard, 2003). Moreover, the combination of salinity and waterlogging occurs in coastal salt marshes by daily tides or seasonal flooding. Microbial activity and aerobic metabolism of roots rapidly reduce the oxygen concentration in the soil (Silvestri *et al.*, 2005; Colmer *et al.*, 2013). So that

the reduced availability of oxygen as a final electron acceptor in the mitochondrial electron transport chain limits oxidative phosphorylation (Bailey-Serres and Voesenek, 2008). As a consequence, cells alter their metabolism to increase anaerobic generation of ATP by glycolysis. This has negative implications as the ATP:ADP-ratio is strongly reduced coupled with a reduced pH value and the fermentation of lactate and ethanol (Roberts *et al.*, 1992; Biemelt *et al.*, 1999; van Dongen *et al.*, 2009; Rocha *et al.*, 2010).

Suaeda maritima is a halophyte commonly found on coastal wetlands in the intertidal zone (Clapham *et al.*, 1981). The optimal growth range of *S. maritima* is between 170 and 340 mM NaCl (Flowers, 1972; Yeo and Flowers, 1980; Alhdad *et al.*, 2013). In order to cope with this saline environment and maintain water availability, *S. maritima* adjusts its osmotic potential by controlled uptake of osmotically dominant ions and their compartmentation in vacuoles together with accumulation of compatible solutes, such as proline and glycine betaine in the cytosol (Hall *et al.*, 1978; Yeo and Flowers, 1980; Moghaieb *et al.*, 2004). Apart from its tolerance to high salt concentrations, *S. maritima* is also adapted to temporary flooding and hypoxic conditions in the soil (Colmer and Flowers, 2008). Wetson and Flowers (2010) showed a reduced growth of *S. maritima* under hypoxia when simulating tidal flooding with artificial seawater. Oxygen depletion in combination with salinity also increased the uptake of Na^+ and decreased that of K^+. Analyses of fermentative processes under hypoxic conditions revealed the importance of fermentation to maintain ATP production by glycolysis (Wetson *et al.*, 2012; Colmer *et al.*, 2013).

Metabolic profiling is an important method available to study the responses of plants to abiotic stress (Guy *et al.*, 2008; Shulaev *et al.*, 2008; Kráľová *et al.*, 2012) and can therefore be used to provide insights into metabolic pathways and fluxes during hypoxia. Besides the activation of

fermentative processes and the enhanced ATP-gain through glycolysis under oxygen deficiency, metabolic profiling can be used to identify definite changes of metabolites associated with, for example the tricarboxylic acid (TCA)-cycle and nitrogen metabolism under hypoxic conditions (Roberts *et al.*, 1992; Gibbs and Greenway, 2003; Bailey-Serres and Voesenek, 2008). In omics-based studies on physiological responses to stress, metabolic analyses have been used to investigate the effect of high salt concentrations on glycophytes and adaptive mechanisms used by halophytes (Kim *et al.*, 2006; Sanchez *et al.*, 2007; Sanchez *et al.*, 2011). A metabolite analysis of tobacco plants, treated with different salt concentrations, identified a dose-related effect indicated by 40 dominant metabolites including organic acids/bases, amino acids, carbohydrates along with choline, pyrimidine and purine metabolites (Zhang *et al.*, 2011). Likewise, in a recent study, Richter *et al.* (2015) showed that, a salt-sensitive maize hybrid treated with 50 mM NaCl accumulated glucose, fructose and sucrose in comparison to a resistant hybrid. In both hybrids the metabolites of the TCA-cycle were reduced when treated with increasing salt concentrations. A comparative study between *Arabidopsis thaliana* and the related halophyte *Eutrema salsugineum* (*Thellungiella salsuginea*) demonstrated that despite a few notable differences (in raffinose and secondary metabolites), similar metabolic pathways were regulated by salt stress in the sensitive and tolerant species. However, the quantitative accumulation of metabolites was higher in *Eutrema* than in *Arabidopsis* showing the adaptability of salt tolerant plants *(Lugan et al., 2010).* Amino acids and metabolites of the TCA-cycle significantly decreased in the shoots of *Suaeda salsa* (synonym of *Suaeda maritima* subsp. *salsa*) seedlings treated with 170 mM and 550 mM NaCl, whereas metabolites associated with osmotic stress were increased in roots and shoots (Liu *et al.*, 2013). Knowledge of tolerance mechanisms to salinity and hypoxia in *S. maritima*, as a model halophyte, has increased in the past 40 years. However, a general metabolic analysis that links the physiological response of these two

environmental factors with the metabolic adaptation of *S. maritima* is still missing. In order to fill this gap, *S. maritima* plants were grown in low, medium and high salt concentrations to compare the adaptive mechanisms to deficient, optimal and stressful salt concentrations. Additionally, hypoxic conditions were applied to investigate the effect of hypoxia combined with different salt concentrations on the physiological and metabolic adaptation of *S. maritima*. The aim of this study was to investigate the impact of hypoxia combined with different salt concentrations on the primary metabolism and fermentative processes of *S. maritima*. At the physiological level we wanted to investigate the effect of different salt treatments to the ionic balance and the pigment content of the plant, with a focus on the physiological adaptation mechanisms during hypoxia. Furthermore, we wanted to clarify, on a metabolic level, how the osmotic adjustment under different salt concentration is affected by hypoxia.

MATERIALS AND METHODS

Plant material and cultivation

Seeds of *Suaeda maritima* were collected from the salt marsh at Cuckmere Haven, East Sussex (UK) (TQ515978) in Oct. 2009, cleaned and stored in a dry environment at 4°C. The seeds were germinated on sand irrigated with a one-fourth-strength nutrient solution (composition below). Plants were grown in a growth chamber under controlled conditions with a day/night cycle of 16 h/8 h at 24°C/21°C and a light flux of approximately 280 µmol photons m^2 s^{-1} at shoot level. After two weeks the plants were transplanted into plastic pots designed for gas-flushing treatments containing 4.5 L of an aerated nutrient solution. Per treatment, five biological replicates with four plants per pot were cultivated. The concentration of the nutrient solution was increased stepwise by double increments every second day until the nutrient solution reached full strength. The full-strength nutrient solution had the following composition: 6 mM KNO_3, 4 mM

$Ca(NO_3)_2$, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 4 µM H_3BO_3, 0.7 µM $ZnSO_4$, 0.3 µM $CuSO_4$, 7 µM $MnSO_4$, 10 µM MoO_3, 20 µM $NiSO_4$, 20 µM $Co(NO_3)$, 0.027 mM NaFeEDTA (modified after: Stout and Arnon (1939)). The nutrient solution was changed every three days. After establishing the full-strength concentration of the nutrient solution the plants were divided into three treatments with different salt concentrations.

Plants were grown in full-strength (high-salt treatment), one half (medium-salt treatment) and one tenth (low-salt treatment) diluted artificial seawater (Harvey, 1966) added to the nutrient solution, which remained the same in all treatments. Full-strength artificial seawater had the following composition: 411 mM NaCl, 53.5 mM $MgCl_2$, 28.2 mM Na_2SO_4, 10.2 mM $CaCl_2$, 8.8 mM KCl. The artificial seawater concentration was increased in one tenth increments every second day until the designated concentration was reached.

After four weeks the plants were randomly divided into two groups with a different degree of aeration for five days: normoxia and hypoxia. Under normoxic conditions the nutrient solution was aerated. To establish hypoxic conditions the nutrient solution was mixed with 0.1 % (w/v) agar and flushed with nitrogen for 30 min before use (Wiengweera *et al.*, 1997; Wetson *et al.*, 2012). Containers were topped up to a constant level with deionised water to replace evapotranspirational losses.

At harvest, shoots and roots were separated, roots were rinsed briefly two times for three seconds in distilled water and blotted to remove surface water. Shoots and roots were weighed and frozen in liquid nitrogen. For further analysis the samples were ground with liquid nitrogen and freeze-dried.

Atomic absorption spectrometry

For the cation extraction, approximately 50 mg of freeze-dried plant material was solubilized in 10 mL 69% nitric acid by microwave digestion at 190°C for 25 min (CEM Cooperation, MARS

154 5, Matthews, USA). The solution was filtered and diluted to a final volume of 100 mL. The
155 cation analysis was performed on an atomic absorption spectrometer (Thermo Fisher Scientific,
156 3300 series, Dreieich, Germany). The results were visualized by using SigmaPlot 12 (Systat
157 Software, San Jose, USA).

158 Betacyanin determination

159 Betacyanin was purified by the method of Hayakawa and Agarie (2010) with minor modification.
160 For this purpose 10-40 mg of freeze-dried plant material was extracted in 1 mL of 100% acetone
161 for 30 min at 4°C. After centrifugation at 17,000 x *g* for 15 min at 4°C, the supernatant was
162 discarded. This procedure was repeated with 100% acetone and 100% ethanol to remove
163 chlorophyll, carotenoid, ascorbic acid and tocopherol. To extract betacyanin, the pellet was then
164 re-extracted with 1 mL of 50% ethanol for 30 min at 4°C. After centrifugation at 17,000 x *g* for
165 15 min at 4°C, the absorbance of the supernatant was measured at 538 nm (JASCO International
166 Co. Ltd., V-550, Tokyo, Japan). Betacyanin concentrations were determined using a calibration
167 curve with a betacyanin standard (Sigma-Aldrich, St. Louis, USA).

168 Pigment determination by HPLC

169 The pigment analysis was based on the high performance liquid chromatography (HPLC) method
170 of Pfeifhofer *et al.* (2002). Approximately 10-40 mg ground freeze-dried plant material was
171 extracted with 2 mL 96% ethanol at 4°C. Pigments were separated by reverse phase HPLC on a
172 HiQsil RP18 column (250 mm x 2.1 mm i.d. 5 µm particle size; KYA Tech Corporation, Tokyo,
173 Japan) and detected with a photodiode array which measured peaks at 440 nm. The mobile phase
174 consisted of two components: solvent A, acetonitrile:water:methanol (100:10:5); and solvent B,
175 acetone:ethyl acetate (2:1). A linear gradient of 10% B to 100% B was used for the first 6 min,
176 followed by an isocratic elution of 100% B for the next 6 min. Then a 2 min linear gradient from

100% B to 10% B followed by an isocratic elution of 10% B for 5 min. The solvent flow rate was 0.4 mL min^{-1}. The injection volume for the standard (DHI Water and Environment, Denmark) was 20 µL and for the samples 5 µL. Calibration curves of the standards were constructed to calculate the concentration of the pigments in the samples.

Glycine betaine detection and determination by 1H-NMR

Glycine betaine was detected and quantified in the plant extracts using 1H-NMR. 50 mg of freeze-dried plant material were suspended in 1 mL of pure ethanol and thoroughly shaken. Suspensions were heated at 85°C until ethanol was completely evaporated. The residues, re-suspended with 1 mL D2O 99.9% containing 500 µM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) as an internal standard, were shaken at 4°C for 1 h. Homogenates were clarified by centrifugation: 15,000 x *g*, 4°C, 20 min before transfer into the NMR tubes (internal diameter: 5 mm). DSS was used as a reference both for determination of chemical shifts and quantification of the signal of interest. 1H-NMR spectra were recorded on a Bruker NMR Avancer III spectrometer operating at a frequency of 400 MHz. At an ambient probe (QNP type) temperature, a FID of 32 K using a spectral width of 6024 Hz induced a resolution of 0.368 Hz per point. Brucker "presat.au" was used for solvent pre-saturation. The processing of the spectra was carried out using MestReNova software. Characteristic 1H chemical shifts of glycine betaine in D_2O were 3.215 ppm (-[N(CH3)3], 9 protons) and 3.853 ppm ([-CH2-], 2 protons) in reference to external calibration curves performed with pure reference glycine betaine.

Amino acids determination by UPLC

The individual free amino acid content was determined from freeze-dried tissues using the method described by Renault *et al.* (2010). Amino acids were first extracted from approximately 10 mg of dry powder with a mixture of methanol–chloroform–water containing a known

concentration of DL-3-aminobutyric acid (BABA) and adonitol as internal standards. Amino acids were then derivatized with a Waters kit (Waters Corporation, Milford, MA, USA) for analysis by UPLC1–DAD (Waters Corporation). Individual amino acids were identified by co-chromatography with pure synthetic compounds (Sigma–Aldrich, St. Louis, MO, USA) and quantified with respect to the BABA signal and individual external calibration curves. Amino acid concentrations were expressed in µmol g^{-1} DW.

LMW NSCs, polyalcohols and organic acids determination by GC-FID

NSCs and organic acids were analyzed by GC-FID according to the modified method of Adams *et al.* (1999). Fifty microliters of the polar extract prepared for amino acid determinations were dried in a vacuum concentrator. The dry residue was dissolved in 50 µL of 20 mg mL^{-1} methoxyamine hydrochloride in pyridine, at 30°C for 90 min. Then 50 µL of N,O-Bis(trimethylsisyl)trifluoroacetamide was added and samples were shaken at room temperature overnight. One microliter of the mixture was injected in a split mode (1:25) at 260°C on a DB5 column (30 m × 0.32 mm × 0.25 µm). The FID detector was set at 300°C. The gas carrier was helium at a flow rate of 1 mL min^{-1}. Oven temperature was programmed as follows: 5 min at 70°C, 6°C min^{-1} until 142°C, 2°C min^{-1} until 222°C, 1°C min^{-1} until 242°C, 20°C min^{-1} until 260°C, 2 min at 260°C, 25°C min^{-1} until 300 C and finally 5 min at 300°C. Metabolites were identified by comparison of sample chromatograms to standard mixtures of known concentration and quantified in absolute amount after normalization against internal standards (adonitol) and plant material dry weight (µmol g^{-1} DW).

Statistical analyses and data visualization

Principal component analysis (PCA) was carried out using the statistical software Analyse-it (Analyse-it Software, Ltd., Leeds LS3 1HS, United Kingdom) for Microsoft Excel (2013,

Microsoft Corporation, Redmont, WA, USA). For statistics and heatmaps (supplement), data were $\log_{10}$-transformed and centred (van den Berg *et al.*, 2006). For statistical analysis, two-way ANOVA and Holm-Sidak test ($P \leq 0.05$) were performed with SigmaPlot 12.5 (Systat Software, San Jose, USA). The relative mean responses of metabolites, heatmaps and the results of statistics are available in the supplement. Heatmaps were created with the MultiExperiment Viewer (MeV 4.9, http://www.tm4.org/mev.htmL) by using Pearson's correlation and complete linkage.

RESULTS

Plant growth and pigment profiles

S. maritima plants were grown in low-, medium- and high-salt concentrations prepared with artificial sea water. After 5 days of hypoxia, none of the plants showed any indication of reduced vitality (Fig. 1). Plants grown in low-salt medium showed reduced growth compared to plants grown in medium- and high-salt concentrations under normoxic conditions. Under hypoxia the growth rate was reduced for plants treated with high-salt concentration. Low- and medium-salt concentrations showed no significant difference in growth between normoxic and hypoxic conditions (Table 1).

The concentration of chlorophyll a, chlorophyll b, beta-carotene and betacyanin are shown in Table 2. At low- and medium-salt concentrations no significant difference in the chlorophyll a concentration between hypoxia and normoxia was detected. Only high salinity combined with hypoxia led to a notable decline in the chlorophyll a concentration. This distinct decrease was also observed for chlorophyll b and for beta-carotene. However, there was no significant difference in the betacyanin concentration for any treatment.

Ion concentrations

Figure 2 shows the ion accumulation in shoots and roots of *S. maritima* treated with different salt concentration under normoxic and hypoxic conditions. With an increasing salt concentration in the root medium the sodium concentration in the shoot also increased (Fig. 2A). Furthermore, plants treated with medium- and high-salt concentrations showed a significantly higher sodium concentration under hypoxic conditions. On the contrary, the potassium concentration in the shoots declined with an increasing salt treatment. This effect was enhanced under hypoxic conditions (Fig. 2B). Plants grown under high-salt concentrations combined with hypoxia showed an accumulation of magnesium (Fig. 2C). The calcium concentration in the shoots was increased in plants treated with medium- and high-salt concentrations in combination with hypoxia (Fig. 2D). As shown in Figure 2 the ion concentration in the root was generally lower than in the shoot. Nevertheless, the accumulation pattern for sodium in both plant organs was similar (Fig. 2E). The potassium concentration in the root under normoxic conditions increased with rising salt treatments. However, under hypoxia the potassium concentration remained unchanged (Fig. 2F). Plants grown under medium- and high-salt concentration showed a significantly higher magnesium accumulation compared to plants grown under normoxic conditions (Fig. 2G). Hypoxic conditions led to an increase of calcium in roots of plants treated with medium- and high-salt concentrations (Fig. 2H).

Metabolic alterations

In order to investigate any metabolic adjustment, shoot and root samples of *S. maritima* plants treated with different salt concentrations were sampled after 5 days of normoxia or hypoxia. A total of 33 reliable primary metabolites were detected in shoots and roots of *S. maritima*. Among them, 23 metabolites were identified as amino acids or amino acid related substances, five metabolites were sugars, three metabolites were organic acids or metabolites of the TCA-cycle.

A principal component analysis (PCA) was performed on the basis of this dataset to exemplify complex data and identify similar patterns among the different treatments. Figure 3A shows the PCA for metabolites of the shoots of *S. maritima*. Two different clusters of samples were distinguished by this analysis. Plants treated with low-salt concentration clearly separated from plants treated with medium- and high-salt concentrations. The clustering in these two groups was also shown for normoxic and hypoxic conditions. Nevertheless, three out of five shoot samples from plants treated with high salinity combined with hypoxia strongly separated from the other treatments. In the roots the pattern of metabolites separated into two main groups (Fig. 3B). Normoxic conditions led to a distinct clustering for every salt treatment. Under hypoxic conditions, plants treated with low- and medium-salt concentrations showed a high variation. However, the combination of high-salt concentration and hypoxia was clearly separated from all the other treatments. The pattern of the metabolites in the shoots changed depending on the salt treatment and the oxygen supply in the hydroponic culture (Table 3). Under normoxic conditions, the total amount of free amino acids in the shoots was nearly 110 µmol g^{-1} DW for each salt treatment. Plants exposed to hypoxia generally showed an increased concentration of free amino acids. A maximal concentration of 175 µmol g^{-1} DW free amino acids was accumulated by plants treated with high-salt concentrations combined with hypoxia. Stress-associated metabolites like proline and glycine betaine, showed an increased accumulation with increasing salt treatments. Moreover, under hypoxia, the accumulation of proline was strongly enhanced for medium- and high-salt concentrations. Compared to proline, glycine betaine showed no increased accumulation under hypoxic conditions. However, with a concentration from 305 - 478 µmol g^{-1} DW in the shoot, glycine betaine was the most abundant metabolite in this study. Sucrose also tended to be increased under hypoxic conditions for all treatments. Other sugars and metabolites of the TCA-cycle were unchanged under the differing salt treatments.

In the roots, low oxygen concentration led to a more distinct metabolic response than treatments with different salt concentrations (Table 4). During hypoxia, metabolites of the TCA-cycle accumulated but did not show significant changes with increasing salt treatment. Compared to plants grown under normoxic conditions, the concentration of succinate and citrate was nearly doubled in roots of plants exposed to hypoxia and low- or medium-salt concentrations. Likewise, malate accumulated in the root of plants exposed to hypoxic conditions for each salt treatment. The sucrose and fructose concentration increased under hypoxic conditions compared to normoxia. Glucose and sorbitol did not change significantly, when the root was exposed to hypoxic conditions. However, plants grown at low-salt concentrations showed a significantly lower glucose concentration than plants grown in medium- or high-salt concentrations. In the roots, low concentrations of trehalose were found: the disaccharide concentration increased at medium- and high-salt concentrations combined with hypoxia. As in the shoots, the proline and glycine betaine concentrations were increased in roots under medium- and high-salt treatments. Glycine betaine was the most abundant metabolite in the roots with a maximum concentration of 390 µmol g^{-1} DW at high-salt treatments. The exposure of roots to hypoxic conditions had no effect on the glycine betaine concentration. However, the compatible solute proline showed an increased concentration with increasing salt treatments and enhanced accumulation under hypoxia. In general the total amount of free amino acids was higher during hypoxia than in normoxia. Roots exposed to low oxygen concentrations showed an increased concentration of free amino acids from 31% up to 41%.

DISCUSSION

Physiological impact of salinity in *S. maritima*

Plants grown under normoxic conditions appeared healthy (Fig. 1) and showed no significant difference in the weight increase for medium- and high-salt concentrations (Table 1). However, low-salt concentrations inhibited the growth of *S. maritima* significantly. Plants treated with high-salt concentrations tended to have a slightly reduced growth rate compared to plants treated with medium-salt concentrations. This result is consistent with an early study showing that NaCl stimulates the growth of *S. maritima* optimally at concentrations of 170 to 340 mM (Yeo and Flowers, 1980), which equates to the medium-salt treatment in our experimental design. In our study, an increasing salt concentration did not induce visible stress reactions such as necrosis in *S. maritima* under normoxic conditions. However, high salt concentrations significantly decreased the photosynthetic pigments chlorophyll a, chlorophyll b and beta-carotene concentrations (Table 2), indicating a degradative effect of high-salt concentrations under normoxic conditions. Although, the optimal growth range characterized *S. maritima* as a halophyte, the high Na^+ and Cl^- concentrations in our experimental design may exceed the ability of the cells to compartmentalize these ions in the vacuole, when treated with high-salt concentrations. A high accumulation of these ions in the cytoplasm could then lead to toxic effects by inhibiting the activity of enzymes and protein synthesis (Flowers and Yeo, 1986; Munns, 2002; Flowers *et al.*, 2015).

In shoots and roots the Na^+ concentration increased with an increasing salt treatment (Fig. 2 A, E). Although the external ion concentration in the nutrient solution at the high-salt treatment was 10 times higher compared to the low-salt treatment, the Na^+ concentration of shoots and roots differed by a factor of ~1.6. This is considered as a minor difference in the Na^+ concentration

change between the different treatments and may be explained by a selective uptake of Na^+ under low salinities. Wang *et al.* (2007) proposed, that the Na^+ uptake in *S. maritima* under low salinities is mediated by a high affinity K-type transporter and under high salinity by an AKT1-type channel. The lower Na^+ concentration in the roots compared to the shoots also indicates a selective uptake, rapid translocation and accumulation of Na^+ ions from the roots into the shoots. In contrast to *S. maritima*, salt sensitive plants such as *Zea mays* show a higher accumulation of Na^+ in the root compared to the shoot when treated with high salt concentrations (Cramer *et al.*, 1994; Zörb *et al.*, 2004). The small difference in the Na^+ concentration between the roots and the shoots of *S. maritima* will cause a gradient in the osmotic potential. This gradient allows *S. maritima* to take up water even when the plant is exposed to very high salinity. The transport and the accumulation of water into the shoots is a critical physiological function, because *S. maritima* is able to dilute high ion concentration in leaves by its leaf succulence (Yeo and Flowers, 1980; Hajibagheri *et al.*, 1984).

Physiological impacts of salinity combined with hypoxia

Compared to medium-salt concentrations, *S. maritima* plants treated with low- or high-salt concentration showed a reduced growth under normoxic conditions (Table 1). This decreased growth rate was also present under hypoxic conditions. However, the growth reduction of *S. maritima* treated with suboptimal salt concentrations (low and high salinity) was stronger under hypoxic conditions. Furthermore, the combination of high-salt concentrations and hypoxia resulted in a sharply decreased chlorophyll concentration (Table 2), indicating a severe stress to the photosynthetic apparatus under these conditions.

The physiological response observed in the chloroplasts may be explained by an ionic imbalance. The similar physicochemical structures of Na^+ and K^+ cause a competition of Na^+ with K^+ for

transport sites into the symplast, which may result in K^+ deficiency (Maathuis, 1999). In our experimental design we increased the salt concentration by adding artificial sea water to a hydroponic medium. Therefore, the higher salt treatment contained more Na^+, K^+, Mg^{2+}, Ca^{2+} and Cl^- ions compared to the lower salt treatment, but the ratio of the different ions remained equal. Under normoxic conditions, *S. maritima* plants treated with increasing salt concentrations accumulated more Na^+ and less K^+ in the shoot (Fig. 2 A, B). This observation is consistent with the study of Wetson and Flowers (2010), investigating the ion uptake and accumulation in *S. maritima* plants under normoxic and hypoxic conditions. Under hypoxic conditions this accumulation pattern was enhanced. In the roots Na^+ and K^+ accumulated with increased salt treatment under normoxia (Fig. 2 E, F). The fact that under hypoxic conditions Na^+ increased in the roots and shoots whereas the K^+ concentration only increased in the roots but not in the shoots indicates a predominant transport of Na^+ to the shoot. In contrast to normoxia, *S. maritima* plants exposed to hypoxia with an increasing salt treatment showed no increase of K^+ in the roots. These findings are consistent with the results of a comparative review of salt-sensitive and tolerant species by Barrett-Lennard and Shabala (2013), showing that hypoxia under saline conditions causes a simultaneous increase in Na^+ and Cl^- concentrations and a decrease in K^+ concentration in shoots. The authors postulated that the increased Na^+ concentration may be caused by a limited exclusion of Na^+ through Na^+/H^+ antiporters, which are fueled by the plasma membrane H^+-ATPase. The activity of this proton-pump is pH and ATP dependent (Oh *et al.*, 2010) and may be limited through hypoxia-induced acidification and ATP limitation (Felle, 2005). The decreased K^+ concentration may be explained by a hypoxia-induced depolarization of the membrane causing either increased K^+ efflux via depolarization-activated outward-rectifying (KOR) K^+ channels, or reduced K^+ uptake via inward-rectifying (KIR) K^+ channels (Véry and Sentenac, 2002; Shabala, 2003; Barrett-Lennard and Shabala, 2013). The enhanced Na^+ accumulation and

the reduced K^+ uptake of *S. maritima* plants under hypoxic conditions lead to a reduction of the K^+/Na^+-ratio. This shift of the K^+/Na^+-ratio could further limit the photosynthetic efficiency by accumulation of toxic concentrations of Na^+ in the chloroplasts and a deficiency of K^+ as a cofactor for enzymes (Munns *et al.*, 2006; Chaves *et al.*, 2008), which might be a possible explanation for the decreased chlorophyll concentration in the shoots (Fig. 2 A, B; Table 2).

However, in consideration of *S. maritima* as a halophyte, the sharp decrease of chlorophyll under high salinities combined with hypoxia can in our opinion not alone be explained by the toxic effect of a reduced K^+/Na^+-ratio. The high Na^+ concentrations in these plants combined with hypoxia might induce the closure of the stomata (Clipson, 1987). As a consequence the CO_2/O_2 ratio decreases, limiting the CO_2 supply for photosynthesis (Hernandez *et al.*, 1999). To prevent the accumulation of ROS by an over-reduction of the photosynthetic electron transport, some C3 plants like *S. maritima* might use photorespiration for the dissipation of excess light energy (Osmond and Grace, 1995).The large increase of glutamine and ammonium with a simultaneous decrease of glutamate and serine indicate photorespiration of *S. maritima* when grown under high salinity combined with hypoxia (Table 3). Although further research of the photorespiration in *S. maritima* is still required, the degradation of chlorophyll suggests the formation of ROS and the accumulation of ROS-scavenging pigments like betacyanin, we only found a slight and non-significant increase of betacyanin in *S. maritima*. Hayakawa and Agarie (2010) demonstrated an increase of betacyanin under harsh environmental conditions, inducing photoinhibition. The most likely explanation for the slight increase of betacyanin under hypoxia might be the moderate light strength of 280 µmol photons m^2 s^{-1} we used in our experimental design, which could be insufficient to induce photoinhibition and stimulate the synthesis of betacyanin.

The role of compatible solutes under salinity combined with hypoxia

An increased salt concentration enhanced the accumulation of proline and glycine betaine in the roots and shoots of *S. maritima*. Glycine betaine was the most abundant metabolite in our study. Surprisingly, very high glycine betaine concentrations were found even in plants treated with low-salt concentrations. This result can be explained by the high Na^+ accumulation we found in plants treated with low-salt concentrations (Fig. 2). Halophytes like *S. maritima* take up and compartmentalize high amounts of Na^+ in the vacuole. To stabilize the osmotic equilibrium between the vacuole and the cytoplasm, glycine betaine is accumulated in the cytoplasm (Chen and Jiang, 2010). With increasing salt treatments the glycine betaine concentration was enhanced maximally by two fold at high-salt concentrations. This relatively low increase might be explained by the already constitutively high glycine betaine concentrations at lower salt treatments. These results are consistent with other studies which have shown that the primary function of glycine betaine in *S. maritima* might be cytoplasmic osmotic adjustment, due to its very high abundance, cytoplasmic localization and the enhanced accumulation under high-salt concentrations (Hall *et al.*, 1978; Moghaieb *et al.*, 2004). In roots exposed to hypoxic conditions, we did not observe a significant difference in the glycine betaine concentration compared to normoxic conditions. This can be explained by the location of the glycine betaine synthesis. In higher plants, glycine betaine is synthesized in the chloroplasts and then translocated to the roots (Ashraf and Foolad, 2007). Before hypoxic conditions were established, high amounts of glycine betaine would have been accumulated as a part of the osmotic adaption mechanism. The location of the synthesis and accumulation pattern might explain why hypoxia has no significant effect on the glycine betaine concentration.

In comparison to glycine betaine, proline only showed a relatively low concentration in roots and shoots of *S. maritima* (Tables 3 and 4). However, plants treated with medium- and high-salt

concentrations showed an up to 9 fold enhanced proline accumulation. The relatively low concentration of proline in comparison to glycine betaine makes it unlikely that proline plays a major role in the osmotic adjustment of *S. maritima*. However, the strong induction under medium and high-salt treatments indicate that proline might be involved in the protection of cellular structures against toxic salt concentrations or in the redox buffering system of the plant (Yancey, 2005; Verslues and Sharma, 2010).

In the shoots, plants treated with medium- and high-salt concentrations showed an enhanced proline accumulation (Table 3) under hypoxic conditions, indicating a stimulatory effect of hypoxia on the accumulation of proline under salt-stressed conditions. Generally, the accumulation of proline during osmotic stress is caused by an enhanced synthesis in the cytoplasm or plastids and by a reduced degradation in the mitochondria (Elthon and Stewart, 1981; Rayapati *et al.*, 1989; Szoke *et al.*, 1992; Verbruggen and Hermans, 2008). It is suggested, that the proline synthesis might be involved in the oxidative pentose phosphate pathway by consuming NADPH generated in the production of ribulose-5-phosphate (Verslues and Sharma, 2010). This link might be beneficial for the plant because CO_2, which is released in the oxidative pentose phosphate pathway, can be re-assimilated in the chloroplasts. During stress-induced stomatal closure, this mechanism allows continued carbon reduction and may prevent photoinhibition and the accumulation of ROS in the chloroplast (Hare et al., 1998; Verslues and Sharma, 2010). The roots of *S. maritima* generally showed a lower concentration of proline than the shoots. However, the accumulation pattern was similar to the shoots with an enhanced accumulation when exposed to hypoxia (Table 4). The fact that the enhanced accumulation of proline under hypoxic conditions was only found under medium- and high-salt concentrations, indicates that the combination of these two factors induced a stress for the plant. The function of proline under these conditions might be the scavenging of ROS or to buffer the pH and the redox

status of the stressed cells (Verbruggen and Hermans, 2008). To synthesize one molecule of proline by a glutamate-derived pathway, one molecule of ATP and 2 molecules of NADPH are needed (Zhang *et al.*, 1995; Hare *et al.*, 1998; Verbruggen and Hermans, 2008). This high energy demand and the need of reducing equivalents make it unlikely that this pathway of proline synthesis takes place in roots under hypoxic conditions. We therefore suggest, that in *S. maritima* either proline is synthesized in the shoot and then translocated into the roots, or it might be synthesized by an ornithine driven pathway, which is suggested to play an important role in proline accumulation during salt stress (Roosens, 1998). The enhanced concentration of ornithine and of arginine, which is a precursor in the synthesis of ornithine, indicate that this pathway might play a role in the accumulation of proline. The enhanced accumulation under hypoxia may also be caused by a disturbed proline catabolism. The degradation of proline to glutamate via proline dehydrogenase consumes oxygen, which is limited under hypoxic conditions (Huang and Cavalieri, 1979; Elthon and Stewart, 1981). The most likely explanation for the enhanced accumulation under hypoxia is therefore, that the synthesis or the translocation of proline is induced by the medium- and high-salt concentration. As soon as the plant is exposed to hypoxia, the accumulated proline will then no longer be catabolized, which leads to an enhanced proline concentration under oxygen depletion (Fig. 4). The high accumulation of proline might also be beneficial for the recovery after hypoxic conditions. Once the plant is again exposed to normoxic conditions, the degradation of proline again produces high amounts of reduction equivalents which can be used in the reactivated TCA-cycle.

Metabolic adjustments under salinity and hypoxia

The metabolic adjustment of *S. maritima* plants imposed to hypoxia was more prominent in the roots than in the shoots (Tables 3 and 4). Hypoxia induced the accumulation of citrate, malate and succinate (Fig. 4). The accumulation of intermediates of the TCA-cycle during oxygen

depletion was also observed by several other studies (Roberts *et al.*, 1992; Biemelt *et al.*, 1999; van Dongen *et al.*, 2009; Rocha *et al.*, 2010); however, the metabolic pathways causing this accumulation are still uncertain. Metabolite and enzyme-assays of Rocha *et al.* (2010) suggest that alanine aminotransferase provides a link between glycolysis and certain TCA-cycle metabolites during hypoxia. Consistent with this findings, *S. maritima* shows an accumulation of alanine and γ-aminobutyric acid (GABA) during hypoxia. The accumulation of alanine during hypoxia is known in a broad variety of plants (Reggiani *et al.*, 2000; Sousa and Sodek, 2003; Miyashita and Good, 2008; Salvatierra *et al.*, 2016) and is apparently an adaptive mechanism of root cells to regulate the accumulation of pyruvate during oxygen depletion (Fig. 4) (Rocha *et al.*, 2010). The availability of pyruvate is an important factor to control the respiration during hypoxia (Zabalza *et al.*, 2009). Low pyruvate levels limit the respiration rate and help plants to minimize their oxygen demand. Therefore, the high accumulation of alanine in *S. maritima* is another part of its adaptive mechanism to hypoxia. In contrast to ethanol and lactate fermentation, which were already examined in *S. maritima* during hypoxia (Wetson *et al.*, 2012), alanine fermentation has no toxic side effects but does not regain any reducing equivalents. A possible alternative for regaining these reducing equivalents could be the regeneration of NAD^+ via malate dehydrogenase (MDH) (Rocha *et al.*, 2010). In the reverse reaction MDH catalyses the reduction of oxaloacetate to malate using NADH as a co-substrate. This reaction might explain the malate accumulation in our experiment and is also consistent with a previous study of *S. maritima* showing an upregulation of four malate dehydrogenases during hypoxia (Wetson *et al.*, 2012).

The accumulation of GABA is also a common phenomenon among different plant species under hypoxic conditions (Roberts *et al.*, 1992; Miyashita and Good, 2008; Wang *et al.*, 2014). It has been shown that the synthesis of GABA by glutamate decarboxylase can regulate the cytoplasmic pH, since the formation of one GABA molecule consumes one proton (Shelp, 1999). The very

high lactate fermentation rate of *S. maritima* described by Colmer *et al.* (2013) would cause an acidosis and can only be maintained by pH regulatory mechanisms like the formation of GABA. However, we only measured a distinct increase of GABA in roots of *S. maritima* when grown under high salinity combined with hypoxia. The formation of GABA under high salinity may be explained by an osmoregulatory function (Shelp, 1999). It has also been shown that GABA catabolism plays an important role in linking the C and N metabolism in roots of NaCl-stressed *Arabidopsis (Renault et al., 2010).* In the mitochondria GABA can be converted via GABA-transaminase (GABA-T) into succinic semialdehyde (SSA), which is then oxidized by the succinic semialdehyde dehydrogenase (SSADH) into succinate. An increased salt treatment had no significant effect on the succinate formation in the roots of *S. maritima*. However, plants exposed to hypoxia showed a sharp increase of succinate in the roots (Fig. 4). The metabolic pathways leading to an accumulation of succinate during hypoxia are controversial. Further research is needed to clarify whether the succinate accumulation is mediated through the GABA shunt (Shelp, 1999) or the activation of 2-oxogluterate dehydrogenase and succinate-CoA ligase forming succinate from 2-oxoglutarate (Rocha *et al.*, 2010). This partial flux of the TCA-cycle during hypoxia may be beneficial for the plant because the succinate-CoA ligase activity provides ATP under hypoxic conditions.

CONCLUSION

The combination of salinity and hypoxia had a great impact on the metabolism of *S. maritima*, leading to a wide range of physiological and metabolic changes (Fig. 5). Roots exposed to hypoxic conditions showed an increased level of TCA-cycle intermediates such as succinate and malate, indicating a partial flux of the TCA-cycle to regain reduction equivalents. The concentration of free amino acids in roots also increased during hypoxia, suggesting that the formation of alanine might contribute to the tolerance of *S. maritima* by controlling the pyruvate

concentration in the root. A reduced pyruvate level limits the respiration rate and help the plant to minimize its oxygen demand. In the shoots, an altered salt treatment exerted a predominant effect on the metabolic profile of *S. maritima*. Osmoprotectants such as proline and glycine betaine accumulated with an increasing salt treatment. Hypoxic conditions induced an enhanced accumulation of proline under medium- and high-salt concentrations, indicating a metabolic disorder or perturbation under these conditions. Even though, *S. maritima* is tolerant to high salt concentrations and hypoxia, the combination of these two stressors caused an ionic imbalance and photorespiration, indicating a major stress response to these extreme conditions.

ACKNOWLEDMENTS

The authors would like to thank their colleagues Julia Müller and Julia Annika Richter for many useful comments and discussions, which have improved the quality of his paper. This work was supported by a STSM Grant from COST Action FA0901 (Putting Halophytes to Work – From Genes to Ecosystems).

LITERATURE CITED

Adams MA, Chen Z, Landman P, Colmer TD. 1999. Simultaneous determination by capillary gas chromatography of organic acids, sugars, and sugar alcohols in plant tissue extracts as their trimethylsilyl derivatives. *Analytical Biochemistry* **266**: 77–84.

Alhdad GM, Seal CE, Al-Azzawi MJ, Flowers TJ. 2013. The effect of combined salinity and waterlogging on the halophyte *Suaeda maritima*: the role of antioxidants. *Environmental and Experimental Botany* **87**: 120–125.

Ashraf M, Foolad MR. 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany* **59**: 206–216.

Ashraf M, Harris P. 2004. Potential biochemical indicators of salinity tolerance in plants. *Plant Science* **166**: 3–16.

Bailey-Serres J, Voesenek L. 2008. Flooding stress: acclimations and genetic diversity. *Annual Review of Plant Biology* **59**: 313–339.

Baligar VC, Fageria NK, He ZL. 2007. Nutrient use efficiency in plants. *Communications in Soil Science and Plant Analysis* **32**: 921–950.

Barrett-Lennard EG. 2003. The interaction between waterlogging and salinity in higher plants: causes, consequences and implications. *Plant and Soil* **253**: 35–54.

Barrett-Lennard EG, Shabala SN. 2013. The waterlogging/salinity interaction in higher plants revisited - focusing on the hypoxia-induced disturbance to K^+ homeostasis. *Functional Plant Biology* **40**: 872–882.

Biemelt S, Hajirezaei RM, Melzer M, Albrecht G, Sonnewald U. 1999. Sucrose synthase activity does not restrict glycolysis in roots of transgenic potato plants under hypoxic conditions. *Planta* **210**: 41–49.

Chaves MM, Flexas J, Pinheiro C. 2008. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Annals of Botany* **103**: 551–560.

Chen H, Jiang J-G. 2010. Osmotic adjustment and plant adaptation to environmental changes related to drought and salinity. *Environmental Reviews* **18**: 309–319.

Clapham AR, Tutin TG, Warburg EF. 1981. *Excursion flora of the british isles,* 3d ed. Cambridge: Cambridge University Press.

Clipson N. 1987. Salt tolerance in the halophyte *Suaeda maritima* L. Dum. growth, ion and water relations and gas exchange in response to altered salinity. *Journal of Experimental Botany* **38**: 1996–2004.

570**Colmer TD, Flowers TJ. 2008**. Flooding tolerance in halophytes. *New Phytologist* **179**: 964–
571 974.
572**Colmer TD, Pedersen O, Wetson AM, Flowers TJ. 2013**. Oxygen dynamics in a salt-marsh
573 soil and in *Suaeda maritima* during tidal submergence. *Environmental and Experimental*
574 *Botany* **92**: 73–82.
575**Cramer GR, Alberico GJ, Schmidt C. 1994**. Salt tolerance is not associated with the sodium
576 accumulation of two maize hybrids. *Australian Journal of Plant Physiology* **21**: 675–692.
577**de Boer AH, Volkov V. 2003**. Logistics of water and salt transport through the plant: structure
578 and functioning of the xylem. *Plant, Cell and Environment* **26**: 87–101.
579**Elthon TE, Stewart CR. 1981**. Submitochondrial location and electron transport characteristics
580 of enzymes involved in proline oxidation. *Plant Physiology* **67**: 780–784.
581**Felle HH. 2005**. pH regulation in anoxic plants. *Annals of Botany* **96**: 519–532.
582**Flowers TJ. 1972**. Salt tolerance in *Suaeda maritima* (L.) Dum. *Journal of Experimental Botany*
583 **23**: 310–321.
584**Flowers TJ, Colmer TD. 2008**. Salinity tolerance in halophytes. *New Phytologist* **179**: 945–963.
585**Flowers TJ, Munns R, Colmer TD. 2015**. Sodium chloride toxicity and the cellular basis of salt
586 tolerance in halophytes. *Annals of Botany* **115**: 419–431.
587**Flowers TJ, Yeo AR. 1986**. Ion relations of plants under drought and salinity. *Australian*
588 *Journal of Plant Physiology* **13**: 75–91.
589**Ghassemi F, Jakeman AJ, Nix HA. 1995**. *Salinisation of land water resources: human causes,*
590 *axtent, management and case studies*. Oxon: CAB International.
591**Guy C, Kopka J, Moritz T. 2008**. Plant metabolomics coming of age. *Physiologia Plantarum*
592 **132**: 113–116.

Hajibagheri MA, Hall JL, Flowers TJ. 1984. Stereological analysis of leaf cells of the halophyte *Suaeda maritima* (L.) Dum. *Journal of Experimental Botany* **35**: 1547–1557.

Hall JL, Harvey DMR, Flowers TJ. 1978. Evidence for the cytoplasmic localization of betaine in leaf cells of *Suaeda maritima*. *Planta* **140**: 59–62.

Hare PD, Cress WA, van Staden J. 1998. Dissecting the roles of osmolyte accumulation during stress. *Plant, Cell & Environment* **21**: 535–553.

Harvey HW. 1966. *The chemistry and fertility of seawaters*. Cambridge: Cambridge University Press.

Hayakawa K, Agarie S. 2010. Physiological roles of betacyanin in a halophyte, *Suaeda japonica* Makino. *Plant Production Science* **13**: 351–359.

Hernandez JA, Campillo A, Jimenez A, Alarcon JJ, Sevilla F. 1999. Response of antioxidant systems and leaf water relations to NaCl stress in pea plants. *New Phytologist* **141**: 241–251.

Horie T, Karahara I, Katsuhara M. 2012. Salinity tolerance mechanisms in glycophytes: an overview with the central focus on rice plants. *Rice* **5**: 11. doi:10.1186/1939-8433-5-11.

Huang AHC, Cavalieri AJ. 1979. Proline oxidase and water stress-induced proline accumulation in spinach leaves. *Plant Physiology* **63**: 531–535.

Kim JK, Bamba T, Harada K, Fukusaki E, Kobayashi A. 2006. Time-course metabolic profiling in *Arabidopsis thaliana* cell cultures after salt stress treatment. *Journal of Experimental Botany* **58**: 415–424.

Kráľová K, Jampílek J, Ostrovský I. 2012. Metabolomics - useful tool for study of plant responses to abiotic stresses. *Ecological Chemistry and Engineering S* **19**: 133–161.

Liu X, Wu H, Ji C, *et al.* 2013. An integrated proteomic and metabolomic study on the chronic effects of mercury in *Suaeda salsa* under an environmentally relevant salinity. *PLoS ONE* **8**: e64041. doi:10.1371/journal.pone.0064041.

Lugan R, Niogret M-F, Leport L, *et al.* 2010. Metabolome and water homeostasis analysis of *Thellungiella salsuginea* suggests that dehydration tolerance is a key response to osmotic stress in this halophyte. *The Plant Journal* **64**: 215–229.

Maathuis F. 1999. K^+ nutrition and Na^+ toxicity: the basis of cellular K^+/Na^+ ratios. *Annals of Botany* **84**: 123–133.

Miyashita Y, Good AG. 2008. Contribution of the GABA shunt to hypoxia-induced alanine accumulation in roots of *Arabidopsis thaliana*. *Plant and Cell Physiology* **49**: 92–102.

Moghaieb RE, Saneoka H, Fujita K. 2004. Effect of salinity on osmotic adjustment, glycinebetaine accumulation and the betaine aldehyde dehydrogenase gene expression in two halophytic plants, *Salicornia europaea* and *Suaeda maritima*. *Plant Science* **166**: 1345–1349.

Munns R. 2002. Comparative physiology of salt and water stress. *Plant, Cell and Environment* **25**: 239–250.

Munns R, James RA, Lauchli A. 2006. Approaches to increasing the salt tolerance of wheat and other cereals. *Journal of Experimental Botany* **57**: 1025–1043.

Munns R, Tester M. 2008. Mechanisms of salinity tolerance. *Annual Review of Plant Biology* **59**: 651–681.

Oh DH, Lee SY, Bressan RA, Yun DJ, Bohnert HJ. 2010. Intracellular consequences of SOS1 deficiency during salt stress. *Journal of Experimental Botany* **61**: 1205–1213.

Osmond CB, Grace SC. 1995. Perspectives on photoinhibition and photorespiration in the field: quintessential inefficiencies of the light and dark reactions of photosynthesis? *Journal of Experimental Botany* **46**: 1351–1362.

Pfeifhofer HW, Willfurth R, Zorn M, Kranner I. 2002. Analysis of chlorophylls, carotenoids, and tocopherols in lichens. In: Kranner IC, Beckett RP, Varma AK, eds. *Protocols in*

Lichenology: Culturing, Biochemistry, Ecophysiology and Use in Biomonitoring. Berlin, Heidelberg: Springer Berlin Heidelberg, 363–378.

Rayapati PJ, Stewart CR, Hack E. 1989. Pyrroline-5-carboxylate reductase is in pea (*Pisum sativum* L.) leaf chloroplasts. *Plant Physiology* **91**: 581–586.

Reggiani R, Nebuloni M, Mattana M, Brambilla I. 2000. Anaerobic accumulation of amino acids in rice roots: role of the glutamine synthetase/glutamate synthase cycle. *Amino Acids* **18**: 207–217.

Renault H, Roussel V, El Amrani A, *et al.* 2010. The *Arabidopsis* pop2-1 mutant reveals the involvement of GABA transaminase in salt stress tolerance. *BMC Plant Biology* **10**: 20. doi:10.1186/1471-2229-10-20.

Richter JA, Erban A, Kopka J, Zörb C. 2015. Metabolic contribution to salt stress in two maize hybrids with contrasting resistance. *Plant Science* **233**: 107–115.

Roberts JKM, Hooks MA, Miaullis AP, Edwards S, Webster C. 1992. Contribution of malate and amino acid metabolism to cytoplasmic pH regulation in hypoxic maize root tips studied using nuclear magnetic resonance spectroscopy. *Plant Physiology* **98**: 480–487.

Rocha M, Licausi F, Araujo WL, *et al.* 2010. Glycolysis and the tricarboxylic acid cycle are linked by alanine aminotransferase during hypoxia induced by waterlogging of *Lotus japonicus*. *Plant Physiology* **152**: 1501–1513.

Roosens NH. 1998. Isolation of the ornithine-delta-aminotransferase cDNA and effect of salt stress on its expression in *Arabidopsis thaliana*. *Plant Physiology* **117**: 263–271.

Salvatierra A, Pimentel P, Almada R, Hinrichsen P. 2016. Exogenous GABA application transiently improves the tolerance to root hypoxia on a sensitive genotype of *Prunus* rootstock. *Environmental and Experimental Botany* **125**: 52–66.

Sanchez DH, Pieckenstain FL, Escaray FJ, *et al.* 2011. Comparative ionomics and metabolomics in extremophile and glycophytic *Lotus* species under salt stress challenge the metabolic pre-adaptation hypothesis. *Plant, Cell & Environment* **34**: 605–617.

Sanchez DH, Siahpoosh MR, Roessner U, Udvardi M, Kopka J. 2007. Plant metabolomics reveals conserved and divergent metabolic responses to salinity. *Physiologia Plantarum* **132**: 209–219.

Shabala S. 2003. Regulation of potassium transport in leaves: from molecular to tissue level. *Annals of Botany* **92**: 627–634.

Shelp B. 1999. Metabolism and functions of gamma-aminobutyric acid. *Trends in Plant Science* **4**: 446–452.

Shulaev V, Cortes D, Miller G, Mittler R. 2008. Metabolomics for plant stress response. *Physiologia Plantarum* **132**: 199–208.

Silvestri S, Defina A, Marani M. 2005. Tidal regime, salinity and salt marsh plant zonation. *Estuarine, Coastal and Shelf Science* **62**: 119–130.

Smedema LK, Shiati K. 2002. Irrigation and salinity: a perspective review of the salinity hazards of irrigation development in the arid zone. *Irrigation and Drainage Systems* **16**: 161–174.

Sousa C de, **Sodek L. 2003**. Alanine metabolism and alanine aminotransferase activity in soybean (*Glycine max*) during hypoxia of the root system and subsequent return to normoxia. *Environmental and Experimental Botany* **50**: 1–8.

Stout PR, Arnon DI. 1939. Experimental methods for the study of the role of copper, manganese, and zinc in the nutrition of higher plants. *American Journal of Botany* **26**: 144–149.

686**Szoke A, Miao GH, Hong Z, Verma DP. 1992**. Subcellular location of delta-pyrroline-5-
687 carboxylate reductase in root/nodule and leaf of soybean. *Plant Physiology* **99**: 1642–1649.
688**van den Berg RA, Hoefsloot HCJ, Westerhuis JA, Smilde AK, van der Werf, Mariët J.**
689 **2006**. Centering, scaling, and transformations: improving the biological information content
690 of metabolomics data. *BMC Genomics* **7**: 142. doi:10.1186/1471-2164-7-142.
691**van Dongen JT, Fröhlich A, Ramírez-Aguilar SJ, *et al.* 2009**. Transcript and metabolite
692 profiling of the adaptive response to mild decreases in oxygen concentration in the roots of
693 arabidopsis plants. *Annals of Botany* **103**: 269–280.
694**Verbruggen N, Hermans C. 2008**. Proline accumulation in plants: a review. *Amino Acids* **35**:
695 753–759.
696**Verslues PE, Sharma S. 2010**. Proline metabolism and its implications for plant-environment
697 interaction. *The Arabidopsis book / American Society of Plant Biologists* **8**: e0140.
698 doi:10.1199/tab.0140.
699**Véry A-A, Sentenac H. 2002**. Cation channels in the *Arabidopsis* plasma membrane. *Trends in*
700 *Plant Science* **7**: 168–175.
701**Wang C, Fan L, Gao H, *et al.* 2014**. Polyamine biosynthesis and degradation are modulated by
702 exogenous gamma-aminobutyric acid in root-zone hypoxia-stressed melon roots. *Plant*
703 *Physiology and Biochemistry* **82**: 17–26.
704**Wang S-M, Zhang J-L, Flowers TJ. 2007**. Low-affinity Na^+ uptake in the halophyte *Suaeda*
705 *maritima*. *Plant Physiology* **145**: 559–571.
706**Wetson AM, Flowers TJ. 2010**. The effect of saline hypoxia on growth and ion uptake in
707 *Suaeda maritima*. *Functional Plant Biology* **37**: 646.

Wetson AM, Zörb C, John EA, Flowers TJ. 2012. High phenotypic plasticity of *Suaeda maritima* observed under hypoxic conditions in relation to its physiological basis. *Annals of Botany* **109**: 1027–1036.

Wiengweera A, Greenway H, Thomson CJ. 1997. The use of agar nutrient solution to simulate lack of convection in waterlogged soils. *Annals of Botany* **80**: 115–123.

Yancey PH. 2005. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *Journal of Experimental Biology* **208**: 2819–2830.

Yeo AR, Flowers TJ. 1980. Salt tolerance in the halophyte *Suaeda maritima* L. Dum: Evaluation of the effect of salinity upon growth. *Journal of Experimental Botany* **31**: 1171–1183.

Zabalza A, van Dongen JT, Fröhlich A, *et al.* 2009. Regulation of respiration and fermentation to control the plant internal oxygen concentration. *Plant Physiology* **149**: 1087–1098.

Zhang C-s, Lu Q, Verma DPS. 1995. Removal of feedback inhibition of Δ^1-pyrroline-5-carboxylate synthetase, a bifunctional enzyme catalyzing the first two steps of proline biosynthesis in plants. *Journal of Biological Chemistry* **270**: 20491–20496.

Zhang J, Zhang Y, Du Y, Chen S, Tang H. 2011. Dynamic metabonomic responses of tobacco (*Nicotiana tabacum*) plants to salt stress. *Journal of Proteome Research* **10**: 1904–1914.

Zörb C, Schmitt S, Neeb A, Karl S, Linder M, Schubert S. 2004. The biochemical reaction of maize *(Zea mays* L.) to salt stress is characterized by a mitigation of symptoms and not by a specific adaptation. *Plant Science* **167**: 91–100.

Zörb C, Senbayram M, Peiter E. 2014. Potassium in agriculture - status and perspectives. *Journal of Plant Physiology* **171**: 656–669.

LIST OF FIGURES

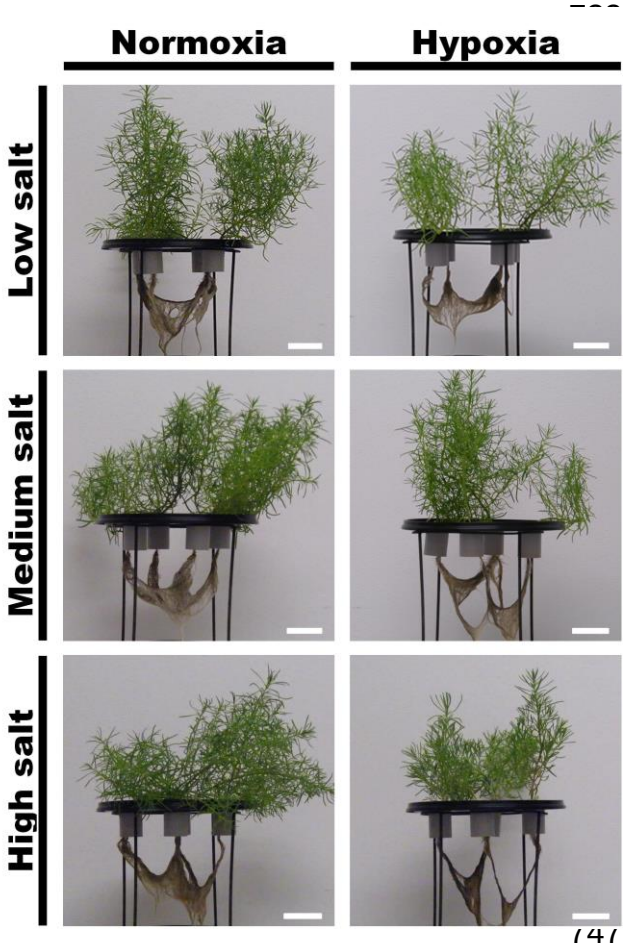
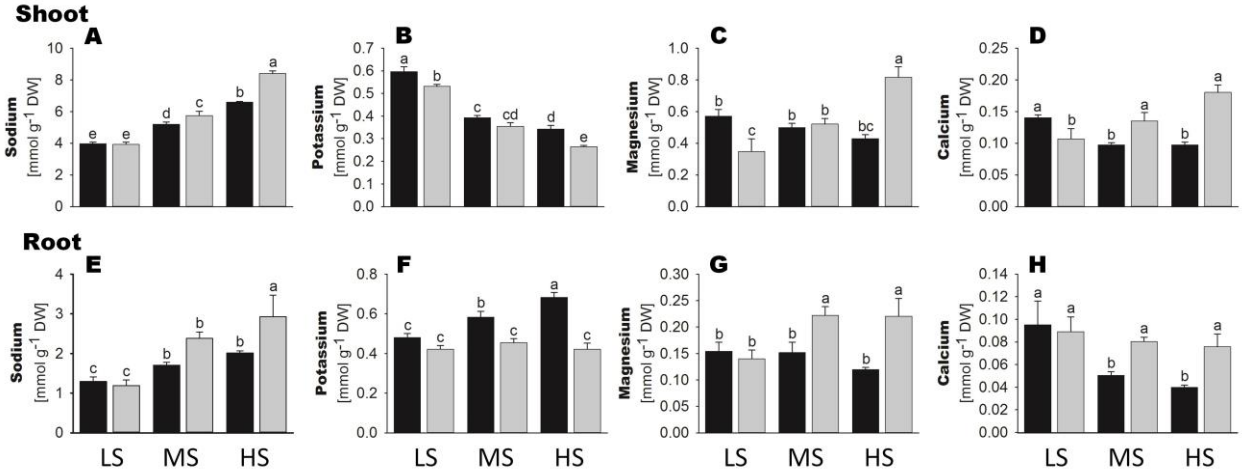


Fig. 1. Phenotype of *S. maritima* grown under different salt concentrations and normoxic and hypoxic conditions. Plants grown for six weeks in a hydroponic culture with low-, medium-, and high-salt concentrations (n = 5). Normoxic and hypoxic conditions were imposed for 5 days (Scale bar = 5 cm).



755

756**Fig. 2. Ion concentrations in the shoots and roots of *S. maritima*.** Quantification of sodium (A
757and E), potassium (B and F), magnesium (C and G) and calcium (D and H) grown under low-salt
758(LS), medium-salt (MS) and high-salt (HS) concentrations. Roots were under normoxic (black)
759or hypoxic (grey) conditions for 5 days. Values are presented as means ± SE of five replicates.
760Different letters indicate significant differences of the means according to post-hoc Holm-Sidak
761test ($P \leq 0.05$).

762

763

764

765

766

767

768

769

770

771

772

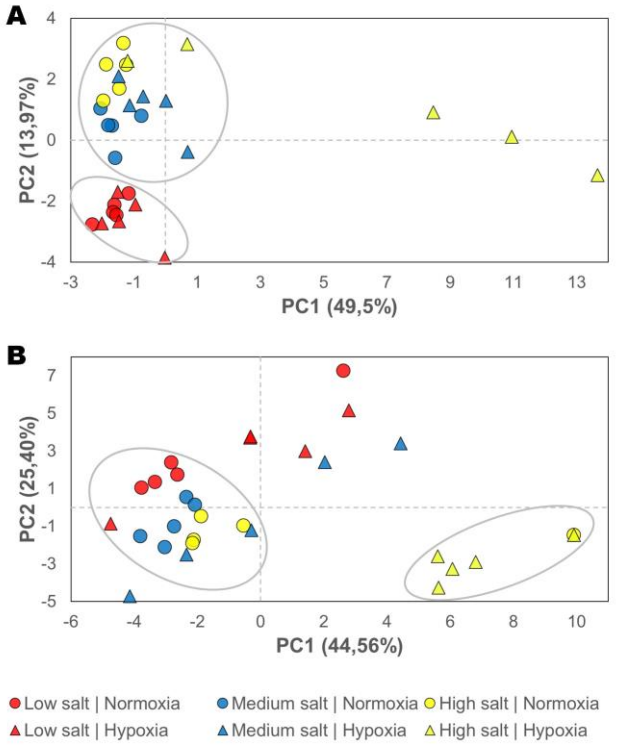
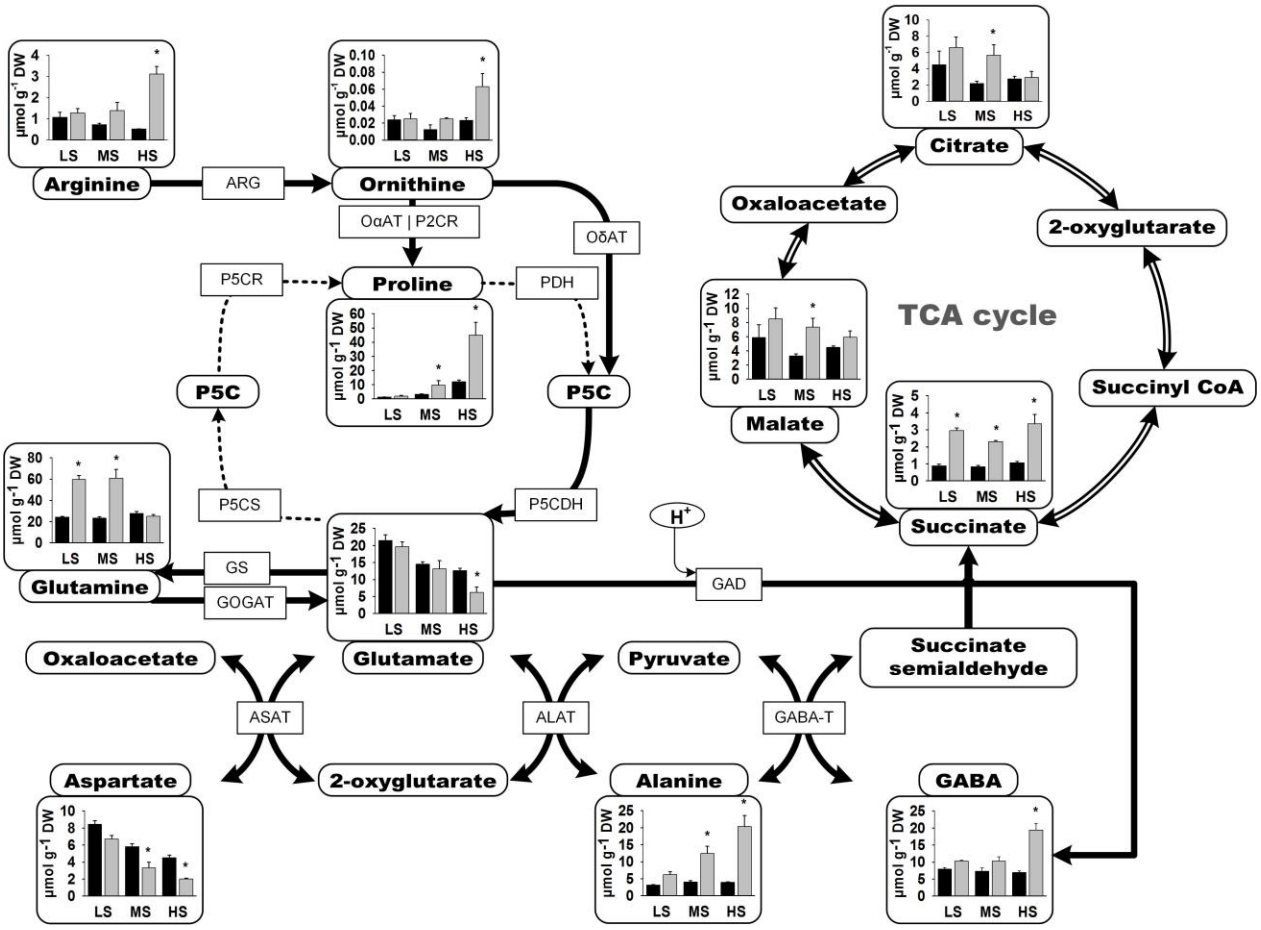


Fig. 3. Principal component analysis (PCA) showing the pattern of metabolic acclimation of *S. maritima* to different salt concentration and hypoxia in the shoots (A) and roots (B). The PCA was performed with 32 metabolites**.** Samples showing cluster patterns are marked with circles (n = 5).



796

797**Fig. 4. Selected metabolites of the TCA-cycle and the GABA shunt in roots of *S. maritima*.**
798Plants were grown under low-salt (LS), medium-salt (MS) and high-salt (HS) concentrations.
799Normoxic (black) and hypoxic (grey) conditions were imposed for 5 days. Values are presented
800as means ± SE of five replicates. Marked hypoxic bars (*) were significantly different from the
801respective normoxic treatment ($P \leq 0.05$). Dotted lines illustrate inhibited pathways under
802hypoxic conditions. ALAT, alanine aminotransferase; ARG; arginase, ASAT, aspartate
803aminotransferase; GABA, gamma-aminobutyric acid; GABA-T, GABA-transaminase; GAD,
804glutamate decarboxylase; GOGAT, GS, glutamine synthetase; PDH, proline dehydrogenase;
805P5C, Δ^1-pyrroline-5-carboxylate; P5CDH, P5C dehydrogenase; P5CR, P5C reductase; P2RC,
806P2C reductase; P5CS, P5C synthase; OαAT; ornithine-α-aminotransferase; OδAT, ornithine-δ-
807aminotransferase.

808

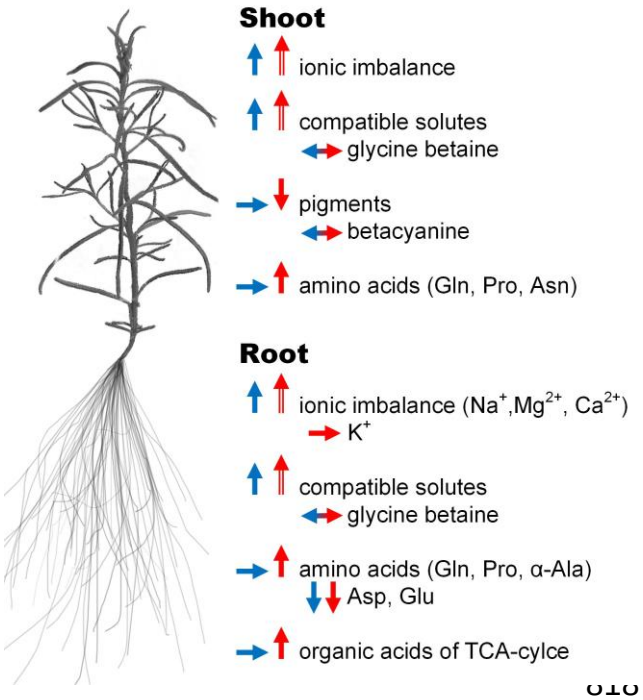


Fig. 5. Schematic summary of the physiological and metabolic adjustment of *S. maritima* to salinity combined with hypoxia. The adjustment of *S. maritima* to different oxygen levels are represented by a blue arrow for normoxia and by a red arrow for hypoxia. The orientation of the arrows imply the physiologic and metabolic changes with an increasing salt treatment: up, increasing level; down, decreasing level; horizontal, steady level. The length of the red arrows represent the magnitude of physiologic and metabolic changes compared to normoxia. Arrows pointing in both directions illustrate no differences between normoxic and hypoxic conditions.

Table 1. Fresh weight increase of *S. maritima* plants under different salt concentrations and normoxic and hypoxic conditions. Total weight increase per plant at 5 days of stress duration. Low-salt, 10% artificial sea water (ASW); medium-salt, 50% ASW; high-salt, 100% ASW. Values are presented as means ± SE of five replicates. Different letters indicate significant difference of the means according to post-hoc Holm-Sidak test ($P \leq 0.05$).

Treatment	Fresh weight increase per plant [g] ± SE	
	Normoxia	Hypoxia
Low-salt	5.8 ± 0.9 b	4.6 ± 0.5 b
Medium-salt	9.3 ± 0.7 a	10.7 ± 1.4 a
High-salt	8.8 ± 0.7 a	2.0 ± 0.4 c

Table 2. Pigment concentration in the shoots of *S. maritima* grown under different salt concentrations and hypoxia. Normoxic or hypoxic conditions were imposed for 5 days. Low-salt, 10% artificial sea water (ASW); medium-salt, 50% ASW; high-salt, 100% ASW. Values are presented as means ± SE of five replicates. Different letters indicate significant difference of the means according to post-hoc Holm-Sidak test ($P \leq 0.05$).

Treatment		Chlorophyll a	Chlorophyll b	Beta-Carotene	Betacyanin
		[mmol g^{-1} DW]	[mmol g^{-1} DW]	[mmol g^{-1} DW]	[µmol g^{-1} DW]
Low-salt	Normoxia	96.6 ± 4.2 a	30.1 ± 1.3 a	9.7 ± 0.3 a	15.1 ± 2.1 a
	Hypoxia	92.1 ± 5.1 a	26.1 ± 1.5 b	9.2 ± 0.6 a	15.5 ± 2.3 a
Medium-salt	Normoxia	86.5 ± 4.5 ab	26.5 ± 1.2 ab	8.2 ± 0.6 ab	10.6 ± 2.9 a
	Hypoxia	74.6 ± 3.8 b	22.3 ± 1.2 c	7.5 ± 0.4 b	12.0 ± 0.9 a
High-salt	Normoxia	75.7 ± 5.9 b	21.7 ± 1.6 c	5.7 ± 0.5 b	13.7 ± 1.6 a
	Hypoxia	14.9 ± 3.7 c	4.5 ± 1.1 d	0.8 ± 0.2 c	16.2 ± 1.2 a

Table 3. Concentration (µmol g^{-1} DW) of metabolites in the shoots of *S. maritima* under different salt and normoxic or hypoxic conditions. Low-salt, 10% artificial sea water (ASW); medium-salt, 50% ASW; high-salt, 100% ASW. ΣAA, total amino acid concentration. Different letters indicate significant difference of the means according to post-hoc Holm-Sidak test ($P \leq 0.05$). Marked (*) metabolites were statistically not evaluable (n = 5).

Metabolite	Low-salt		Medium-salt		High-salt	
	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia
Glutamate	24.5 ab	26.3 a	21.3 ab	18.4 b	16.9 b	6.3 c
Glutamine	23.4 ab	29.8 a	23.6 ab	29.2 a	24.4 b	38.3 a
Histidine	0.2 b	0.2 b	0.2 b	0.2 b	0.2 b	2.4 a
Proline	2.5 d	2.8 d	8.6 c	21.7 b	19.8 b	95.1 a
Arginine	0.3 b	0.2 b	0.2 b	0.7 a	0.1 b	1.7 a
Aspartate	16.7 a	17.2 a	16.8 a	12.4 a	15.3 a	3.1 b
Asparagine	1.9 ab	2.2 ab	1.7 b	2.3 ab	1.5 b	4.1 a
Threonine	2.5 a	2.7 a	2.2 a	2.3 a	1.9 b	1.5 c
Isoleucine	3.0 ab	3.9 a	2.1 b	3.6 a	2.7 ab	3.0 ab
Methionine	0.1 b	0.1 b	0.1 b	0.1 b	0.1 b	0.2 a
Lysine	0.3 ab	0.3 ab	0.2 b	0.2 b	0.2 b	0.6 a
Serine	29.4 a	30.4 a	17.6 b	20.4 b	11.1 c	7.5 d
Tryptophan	0.2 b	0.3 b	0.2 b	0.3 b	0.1 b	0.7 a
Tyrosine	0.7 ab	0.7 ab	0.6 b	0.5 b	0.5 b	1.1 a
Phenylalanine	0.6 b	0.7 b	0.6 b	0.7 b	0.5 b	1.5 a
β-Alanine	0.5 b	0.5 b	0.4 b	0.5 b	0.5 b	1.3 a
α-Alanine	10.5 a	10.3 a	13.2 a	10.3 a	12.8 a	3.9 b
Leucine	0.4 b	0.4 b	0.7 ab	0.4 b	0.4 b	0.8 a
Valine	1.3 b	1.4 b	1.5 b	1.4 b	1.4 b	2.3 a
ΣAA	119.0	130.5	111.6	125.7	110.3	175.2
Ammonium	7.8 b	6.6 bc	4.9 c	6.5 bc	5.8 bc	22.3 a
GABA	2.2 a	2.6 a	1.6 a	1.8 a	1.2 ab	0.8 b
Glycine betaine	305.1 c	322.1 c	379.5 b	394.9 b	478.5 a	413.9 b
Sucrose	24.1 a	32.6 a	30.9 a	38.6 a	34.8 a	41.6 a
Glucose	10.1 a	9.6 a	7.9 a	4.2 b	6.0 a	7.5 a
Fructose	9.8 a	9.8 a	7.9 a	4.5 b	7.1 a	7.2 a
Citrate*	1.4	1.9	1.8	2.1	1.7	1.7
Malate*	22.2	23.2	24.6	24.8	25.0	20.7
Succinate	2.0 a	1.6 ab	1.7 ab	2.0 a	2.1 a	1.2 b
Glycerate	1.7 a	2.3 a	1.5 a	1.7 a	1.7 a	2.2 a
Myo-inositol	0.6 a	0.7 ab	0.5 a	0.4 b	0.4 b	0.3 c

Table 4. Concentration (µmol g^{-1} DW) of metabolites in the roots of *S. maritima* under different salt and normoxic or hypoxic conditions. Low-salt, 10% artificial sea water (ASW); medium-salt, 50% ASW; high-salt, 100% ASW. ΣAA, total amino acid concentration. Different letters indicate significant difference of the means according to post-hoc Holm-Sidak test ($P \leq 0.05$). Marked (*) metabolites were statistically not evaluable (n = 5).

Metabolite	Low -salt		Medium-salt		High-salt	
	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia
Glutamate	21.5 a	19.6 a	14.5 a	13.1 a	12.6 a	6.3 b
Glutamine	24.3 b	59.7 a	23.3 b	61.0 a	27.7 b	25.1 b
Histidine	0.8 b	1.0 b	0.4 c	1.1 b	0.8 b	2.1 a
Ornithine	0.024 b	0.031 b	0.021 b	0.025 b	0.023 b	0.063 a
Proline	1.3 e	1.9 e	3.3 d	9.7 c	12.0 b	45.0 a
Arginine	1.1 ab	1.3 ab	0.7 ab	1.4 ab	0.5 b	3.1 a
Aspartate	8.5 a	6.7 a	5.8 a	3.3 b	4.5 b	2.0 c
Asparagine	3.8 b	4.4 ab	3.2 b	4.3 ab	3.2 b	5.6
Threonine*	2.2	2.3	2.0	1.7	2.0	2.0
Isoleucine	0.8 b	1.0 b	0.7 b	0.7 b	0.8 b	1.7 a
Methionine*	0.2	0.2	0.1	0.1	0.1	0.1
Lysine	0.9 b	1.0 b	0.6 c	1.0 b	0.5 c	1.7 a
Serine*	10.9	10.8	10.2	9.5	9.8	8.5
Tryptophan	0.8 b	0.9 b	0.6 b	0.8 b	0.7 b	1.8 a
Tyrosine*	1.9	1.5	1.6	1.4	1.9	1.8
Phenylalanine	0.4 b	0.6 ab	0.3 b	0.4 b	0.4 b	0.7 a
β-Alanine	0.5 b	0.6 b	0.7 b	0.9 b	1.2 a	1.6 a
α-Alanine	3.1 d	6.2 c	4.1 d	12.5 b	3.9 d	20.4 a
Leucine	0.7 b	0.8 b	0.6 b	0.7 b	0.5 b	1.6 a
Valine	1.2 b	1.6 b	1.1 b	1.4 b	1.2 b	3.2 a
ΣAA	84.8	122.2	73.9	125.1	84.3	134.2
Ammonium	5.1 b	9.0 b	7.2 b	10.1 b	13.0 a	17.0 a
GABA	7.9 b	10.3 c	7.3 b	10.3 c	7.0 b	19.4 a
Glycine betaine	205.3 c	204.5 c	325.2 b	353.7 ab	390.1 a	390.2 a
Sucrose	48.1 a	44.5 a	35.1 a	38.8 a	41.5 a	17.1 b
Glucose	5.1 b	5.7 b	11.2 a	14.4 a	13.5 a	18.4 a
Fructose	6.3 c	9.8 b	9.9 b	19.3 a	16.3 a	17.3 a
Sorbitol	2.9 a	2.7 a	2.2 a	2.0 a	1.8 a	0.6 b
Trehalose	0.3 c	0.3 c	0.3 c	0.6 b	0.4 c	1.1 a
Citrate	4.5 a	6.6 a	2.2 b	5.7 a	2.8 b	2.9 b
Malate	5.9 b	8.5 ab	3.3 b	7.4 a	4.5 b	5.9 a
Succinate	0.9 b	3.0 a	0.8 b	2.3 a	1.1 b	3.4 a
Myo-inositol	0.8 a	0.8 a	0.5 c	0.6 bc	0.4 c	0.7 ab

848